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THE RELATION AMONG GONADAL STEROIDS, IMMUNOCOMPETENCE, BODY MASS, AND BEHAVIOR IN YOUNG BLACK-HEADED GULLS (*LARUS RIDIBUNDUS*)

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Abstract.—We experimentally examined the effect of testosterone on the antibody response to a single immunization with sheep red blood cells in young black-headed gulls. This species performs a number of testosterone-mediated elaborate postural displays in social interactions and breeds in dense colonies in which there is a high likelihood of infectious diseases. In young chicks, only one-third were capable of responding to immunization. In the responding chicks, testosterone enhanced antibody titers. Even when antibody responsiveness was measured >1 mo after the termination of hormonal treatment, antibody responses were enhanced in birds treated with androgen but not estrogen. At 9 mo of age, all birds responded to immunization, but there was no effect of testosterone on antibody titers. In these juveniles, frequency of display behavior was negatively related to changes in body mass, which suggests that displaying is energetically costly. Despite decreased body mass, antibody titers were highest in birds that displayed more frequently. This suggests that displaying signals immunocompetence. The results are discussed in relation to the immunocompetence handicap hypothesis and to what is known of the influence of the bursa of Fabricius and social stress on antibody production.

Courtship and agonistic displays are essential for reproduction in virtually all bird species. In males, and in females of some species, testosterone (T) facilitates the expression of these displays (Balthazart 1983; Wingfield et al. 1990). Field studies on birds have shown that males treated with T have greater success in male-male competition and attraction of mates (Moss et al. 1994; Ketterson et al. 1996). However, studies have also shown costs of persistently elevated levels of T due to reduced parental care and reduced survivorship in T-treated birds (Dufty 1989; Wingfield et al. 1990; Moss et al. 1994; Saino et al. 1995; Ketterson et al. 1996). Thus, T secretion is an important factor in mediating a trade-off between sexual display and survival in birds.

Because T simultaneously influences a diverse array of metabolic processes (Mooradian et al. 1987; Ketterson et al. 1996), little is known about how the hormone reduces survivorship. Experimental studies on mammals have shown

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that T suppresses the immune system (Grossman 1985). As a result, individuals may be more vulnerable to disease and parasite infections during the breeding season when individuals are actively involved in courtship and agonistic displays and T levels are high.

A relation between sexual displays and parasite resistance was first proposed by Hamilton and Zuk (1982). Parasite-host coevolution provides a mechanism to maintain heritable variation in resistance among individuals. This variation in resistance can be revealed by the quality of sexual displays because parasites can affect these displays both directly, for example, by deteriorating plumage, and indirectly by interfering with health (Hart 1990; Wedekind 1992). Folstad and Karter (1992) proposed in the immunocompetence handicap hypothesis that T may provide a direct link between sexual display and parasite resistance. The hypothesis is based on the findings that T affects displays positively but at the same time compromises the immune system. They suggest that effects of parasite resistance genes would be reliably revealed by T-induced immunosuppression because only those males that are genetically resistant can remain healthy when acquired immunity is reduced.

The experimental evidence in favor of immunosuppression by T in birds comes mainly from experiments with domestic fowl. In this species, many androgen receptors are found in the bursa of Fabricius, an important organ for the maturation of antibody-producing cells (Gasc and Stumpf 1981; Glick 1983). Experimental treatment of fowl embryos with T causes regression of the bursa and leads to impairment of antibody production (Glick 1983; Ratcliffe 1989). However, these results cannot be taken as general proof for T-induced immunosuppression in birds, because fowl embryos were treated with pharmacological doses of T, whereas not much is known about the effects of physiological levels of T (Norton and Wira 1977). Furthermore, the effect of T on the immune system in young birds may differ from that in adult birds because the bursa regresses before the birds become adult (Glick 1983).

So far, not much is known about the effects of T on the immune system of adult birds. Field studies have shown no consistent correlations between endogenous T levels and parasite infestations (Weatherhead et al. 1993; Saino and Møller 1994). However, within a population individuals may differ in hormone production because of individual differences in social context or quality. The latter will result in individual differences in the extent to which birds are able to produce T without compromising the immune system. As a consequence of this, birds may vary in immune function despite the same production of T, or vice versa. Therefore, such correlations are difficult to interpret if quality differences are unknown. To control for such individual variation, the effect of an increase in T levels was tested experimentally in a field study on barn swallows. In this study, Saino et al. (1995) showed that immunoglobulin levels initially decreased in birds treated with T. Birds recaptured >1 mo after the start of hormonal treatment had higher infestations of ectoparasites than did control birds. However, at this time T-treated birds also had increased levels of immunoglobulin and numbers of eosinophiles despite the increased levels of T. Clearly, more research is

necessary to understand the interactions among T, sexual display, and the immune system.

We studied the effect of T on the immune system in several age classes of the black-headed gull (*Larus ridibundus*) for two primary reasons. First, early in life, immunosuppression would have important functional consequences in this species. Black-headed gull chicks grow up in dense colonies in which they are completely dependent on the parents for food during the first 8 wk after hatching. In the course of the first 2 wk, both parents often leave the territory simultaneously to find food for the chicks. The chicks then have to defend their nest site against intruding chicks and adults by means of T-mediated agonistic behavior (Groothuis 1989; Groothuis and Meeuwissen 1992). This early production of T may affect immune function in an environment in which fecal material, spilled food, and dead young accumulate rapidly. Studies on black-headed gull nestlings have reported that 27%–100% of chicks are infected with *Cryptosporidium baileyi*, a parasite typical for young animals, and one-third infected with *Salmonella* spp. (Literák et al. 1992; Pavlásek 1993). Mortality of chicks has been attributed to these infectious agents (Pavlásek 1993).

Second, many studies on adult birds in which the functional consequences of the expression of sexual displays have been analyzed have investigated species in which T does not play an important role in the expression of these displays (Owens and Short 1995). In adult black-headed gulls, T does play an important role in the expression of some ornaments (brown mask and red bill) and of conspicuous postural displays (van Oordt and Junge 1933; Groothuis and Meeuwissen 1992). For the study of these displays, both males and females are interesting because both sexes acquire the sexual ornaments and perform the same postural displays (van Rhijn 1985). The frequency of these displays shows a spectacular increase in the beginning of the reproductive season, which plays an important role in partner choice and territorial establishment (Tinbergen 1959; Patterson 1965; van Rhijn and Groothuis 1987; Bukacińska and Bukaciński 1994). Some evidence exists that adult black-headed gulls are, compared with other bird species, relatively sensitive to infections with pathogens (Hejlícek and Treml 1995). Consequently, T-induced suppression of immunocompetence in black-headed gulls may have important functional consequences: since the species is long-lived, the increased likelihood of dying because of the effects of immunosuppression would affect future reproductive prospects; based on handicap theory, the expression of T-mediated sexual displays would reliably reveal immunocompetence, thereby making sexual selection on these displays beneficial to females and competent males (Zahavi 1975; Folstad and Karter 1992).

This article addresses the following questions: Do experimentally elevated T levels within the physiological range affect antibody responsiveness of gull nestlings? Does such an effect persist longer than the actual hormonal treatment? Is this effect specifically androgen dependent? Does the effect of T on antibody responsiveness differ with age? What is the relation among the frequency of display, antibody responsiveness, and changes in body mass?

To address these questions, three experiments were done. In the first two ex-

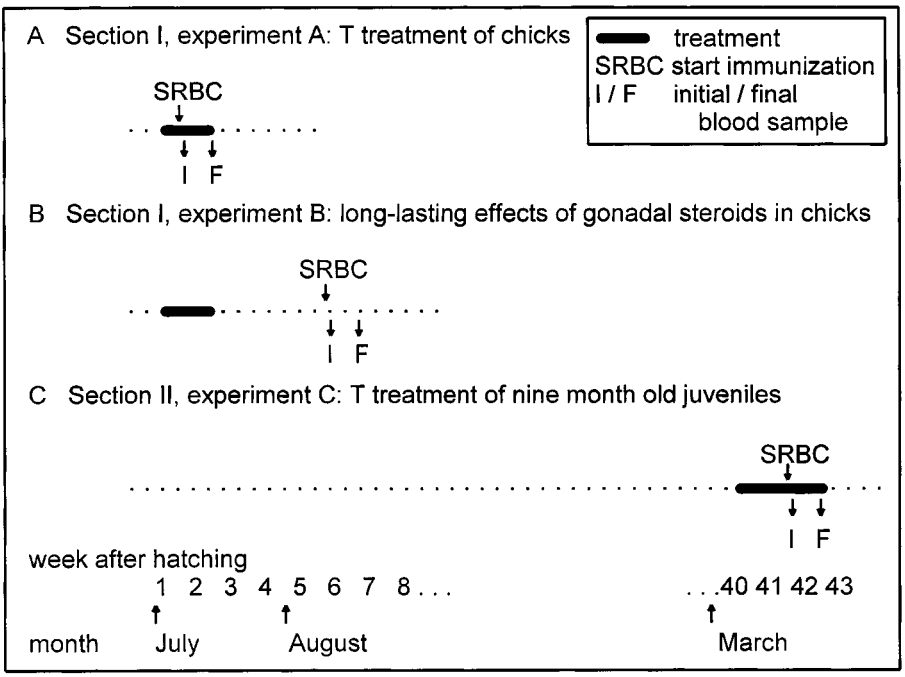

FIG. 1.—Design of the experiments; chicks hatched in the last week of June

periments (see fig. 1*A*, *B*), chicks of 2 wk of age, the age at which they start to become active in display behavior in the field, were treated for 10 d with T (experiment A) or its metabolites: 5α-dihydrotestosterone or 17β-estradiol (experiment B). To analyze the relation among T, antibody responsiveness, display behavior, and changes in body mass in older birds, in the third experiment 9-mo-old gulls were treated with T (fig. 1*C*). As we pointed out earlier, the mechanisms underlying the possible effects of T on the immune system are likely to change with the age of the birds. Therefore, we treat the results in two sections: the first shows the results of early treatment with gonadal steroids; the second shows the results of T manipulation later in life. In investigating hormonal effects later in life, we performed experiments on 9-mo-old juveniles instead of adults because T levels at this age are still very low so that castration is not necessary to produce control birds for hormone experiments. Juvenile birds at the age of 9 mo can be regarded as a model for the adult situation. At the age of 10 mo, endogenous levels of T will increase, and all adult aggressive and courtship behaviors may be performed (Groothuis 1989; Groothuis and Meeuwissen 1992). Furthermore, in the field such birds have been reported to mate and breed (Patterson 1965; Bukacińska and Bukaciński 1993). The effect of T on the immune system was analyzed by measuring antibody production in response to a single injection of sheep red blood cells (SRBC). This is a common method for assessing humoral immunity. The results are discussed in relation to a possible

trade-off between territorial behavior and the immune system in black-headed gull chicks and the immunocompetence handicap theory in juveniles.

TESTOSTERONE TREATMENT OF CHICKS

Design

In this section, we describe the effects of T on immunocompetence early in life. In the field, broods of three or fewer siblings defend nest sites with threat displays and attacks. Chicks never direct aggressive behavior at their siblings. This is also true of chicks in experimental groups of not more than five chicks put together in the first week of their life. In this condition, agonistic behavior and levels of T of the chicks are very low (Groothuis and Meeuwissen 1992).

In experiment A, direct effects of treatment with T on antibody responsiveness were measured during a 10-d treatment period starting 1 wk after hatching of the chicks (see fig. 1*A*). To measure longer-lasting effects of T, the chicks in experiment B were treated as in experiment A, except that the antibody responsiveness was measured >1 mo after termination of hormonal treatment (see fig. 1*B*). Additionally, in experiment B the hormonal specificity of the antibody responsiveness was tested by comparing the effects of T treatment with the effect of two metabolites of T: 17β-estradiol (E) and 5α-dihydrotestosterone (DHT). To test humoral immunity, birds were injected with sheep red blood cells (SRBC), and the increase in antibodies against this antigen over a period of 6 d was measured (Apanius, in press).

Methods

Rearing conditions.—Black-headed gull chicks of 2–3 d of age were collected in June from a large gull colony in the north of the Netherlands and hand reared at the laboratory where they were housed in groups of four or five peers. Groups were held in adjacent cages measuring $85 \times 75 \times 85$ cm. The floors of the cages were covered with straw. Each cage was visually isolated from other cages and contained a thermal lamp providing constant light and a temperature of approximately 37°C in the middle of the cage. After 2 wk, the thermal lamp was replaced by a lamp providing a temperature of 25°C. At this age, the light schedule was changed to 16L:8D. In the dark phase, a small lightbulb provided dimmed light. All lamps were removed from the cages when the chicks were 1 mo old. Strip lighting was used for a 16L:8D schedule after this age. The chicks were individually marked (on the head or back) with rhodamine or picrine (ICN Biochemicals, Cleveland, Ohio; chemicals were dissolved in acetone).

Food and water were available ad lib. During the first 2 wk, chicks were fed with a moistened mixture of trout pellets (Trouvit, Trouw, Gent) and a mixture used for growing chicks (Sivo start, Bogena, Waalwijk). This basic diet was supplemented daily with smelt (*Osmerus eperlanus*) and mashed hard-boiled chicken eggs. At 2 wk of age, the chicks received a diet gradually shifted to dry trout pellets, with egg added twice a week. A vitamin supplement (Calviet,

UTD, Meppel) was added weekly. At 4 mo of age, they were fed egg once every 2 wk.

Treatment.—In experiment A, 9-d-old chicks were implanted with a silicon tube (silicone tubes Medica BV, 's Hertogenbosch; internal diameter = 1 mm, external diameter = 3 mm, length = 12 mm, sealed on both sides with 1 mm of silicon glue) filled with 8 mg of crystalline testosterone (Diosynth, Oss, The Netherlands). The tube was removed after 10 d. Ages at implantation, dosage of T, and duration of implantation were based on the results of an earlier study (A. F. H. Ros, T. G. G. Groothuis, and S. J. Dieleman, unpublished manuscript) showing that T levels (0.4–0.6 ng/mL) induced by the implants were well within the range of naturally occurring T levels in chicks. Chicks reared in seminatural conditions in the laboratory in which many agonistic interactions occur have endogenous T levels of 1 ng/mL (A. F. H. Ros, T. G. G. Groothuis, and S. J. Dieleman, unpublished manuscript). Fifteen chicks were treated with T, and 23 birds served as experimental controls.

In experiment B, 6-d-old chicks received an implant with T or one of its metabolites, E and DHT. The implant was removed after 10 d. A pilot study to measure the effects of different metabolites of T on agonistic behavior of blackheaded gulls had shown that E and DHT diffused from silicon implants much less readily than did T. Therefore, to enhance diffusion of E and DHT, 25-mg crystalline pellets of the T, DHT, and E were used. Similar implants of testosterone propionate were shown in an earlier study to induce T levels within the physiological range and to mediate agonistic behavior (1.45 ng/mL; Groothuis and Meeuwissen 1992). At the time of immunization, a total of 10 T-treated birds, nine DHT-treated birds, five E-treated birds, and 14 control birds were present.

Tubes or pellets were implanted subcutaneously in the neck region, under local anesthesia with lidocaine (Xylocaine, Astra, Rijswijk). When necessary, stitches were used to close the wound. Treatment was terminated by removal of the implant under local anesthesia with lidocaine. Controls were handled and operated as the experimental birds but were not implanted.

Differences between the sexes.—Because we determined sex on the basis of morphological measurements that are reliable only after the birds fledge, it was possible to determine sex only in experiment B. Eighteen males and 20 females were randomly distributed over the experimental groups. In this experiment, no significant effects of sex were found (ANOVA of antibody responses with treatment and sex as factors: sex and sex $\times$ treatment interaction, $P > .10$). Therefore, sex differences are of minor importance in the chick phase.

Radioimmunoassays.—For measuring levels of gonadal steroids, 0.5 mL blood was drawn from the brachial wing vein with a heparin-rinsed needle and syringe within 5 min of capturing the bird. After centrifugation, plasma was stored at $-30°C$ until analyzed. Radioimmunoassays for estimation of testosterone and 17β-estradiol levels were carried out at the Department of Herd Health and Reproduction at the University of Utrecht. Steroids were extracted from the plasma samples with diethyl ether. No further separation of steroids was carried

out. For methods and evaluation of the radioimmunoassays used, see Dieleman et al. (1983) for testosterone and Dieleman and Bevers (1987) for 17β-estradiol.

Previous studies at the Utrecht laboratory showed that in the assay for estimating T levels, the main cross-reactivities with the antiserum were 49.7%, 7.54%, and 3.35% for 5α-dihydrotestosterone, 4-androstene-3β, 17β-diol, and androstenedione, respectively. The interassay coefficient of variation of this assay was 14% (Dieleman et al. 1983). In our assays, lower detection levels of 0.05 ng/mL T and upper detection levels of 4 ng/mL T were used. The main cross-reactivities of the antisera used in the assay for 17β-estradiol were 1.1%, 0.32%, and 0.16% for estrone, estriol, and 17β-estradiol, respectively, and $<0.01\%$ for other steroids tested (according to the manufacturer of the antisera, Coat-A-Count TKE; Diagnostic Products Corporation). Previous studies at the Utrecht laboratory showed that the interassay coefficient of variation of this assay was 8.9% (Dieleman and Bevers 1987). In our assays, lower detection levels of 5 pg/mL E and upper detection levels 400 pg/mL E were used. Measurements for one experiment were made within a single assay to avoid interassay variability.

Immunization and hemagglutination assay.—Sheep red blood cells (SRBC) from one donor sheep were collected in Alsever's solution and used as antigens. The cells were washed three times in phosphate-buffered saline (PBS) and resuspended in PBS at $5 \cdot 10^8$ cells/mL (2% SRBC). Each bird was immunized with the antigen suspension by intraperitoneal injection. The amount of SRBC suspension was adjusted to the size of the birds (Aitken and Parry 1974). In experiment A, birds were immunized on day 4 after T or sham treatment with 0.3 mL SRBC suspension (mean body mass = 150 g). In experiment B, birds were immunized 1 mo after the termination of hormonal treatment with 0.5 mL SRBC suspension (mean body mass = 250 g). Preimmunization blood samples (0.5 mL) were drawn to measure preexisting antibody titers (see Seto and Henderson 1968). Based on prior information of the peak in antibody levels after SRBC immunization in chickens, pheasants, quails (Aitken and Parry 1974), and black-headed gulls (T. G. G. Groothuis, P. Korsten, and L. Zwiggelaar, unpublished data), postimmunization blood samples were drawn 6 d after SRBC injection. Blood was collected and stored in the same way as described in the section on radioimmunoassays.

For the estimation of antibody levels, the following procedure was used. To prevent lysis of sheep red blood cells by complement, the plasma was heated to 56°C for 30 min. Thereafter, plasma was diluted 1:1 in PBS and then serially diluted in PBS in U-shaped microtiter plates (dilution series: 2^1, 2^2, 2^3, 2^4, . . .). An equal volume of 2% SRBC was added to these dilutions, and the plates were incubated at 37°C for 60 min. Titers were scored visually as the highest twofold dilution of plasma showing hemagglutination and represented as integers on a $\log_2$ scale. Measurements of one experiment were done within a single assay to avoid interassay variability. Intra-assay variability was 5.4%. As a measurement of immunocompetence, the antibody titer was calculated as the post- minus pre-immunization score.

Statistical treatment.—Measurements of T levels showed skewed distributions. Therefore, T measurements were $\log(X + 1)$ transformed before calculating parametric statistics (see Zar 1984). After transformation, the data reached the normal distribution. Antibody titers were already expressed on a logarithmic scale and appeared normally distributed. Two-tailed statistics were used with alpha set to 0.05. Two-sample *t*-tests were used for testing single comparisons. In experiment B, ANOVA was used to test the multiple hormonal treatments within one model. Additionally, single comparisons by means of *t*-tests were made to test the experimental groups against the control group. To correct for the increased chance of Type I statistical errors due to multiple testing, *P* values were adjusted for comparison with alpha based on the number of tests, Ntest, carried out: the most significant value was multiplied with Ntest, the second most with Ntest-1, and so forth (our modification of the Bonferroni method of Rice 1989).

RESULTS

Experiment A: Testosterone in Young Chicks

Testosterone levels.—In comparison with untreated birds (control, or C, group), T treatment significantly elevated the T plasma levels (mean ± SE: C group, 0.10 ± 0.06 ng/mL, $N = 9$; T group, 0.26 ± 0.03 ng/mL, $N = 15$; two-sample *t*-test: $t = 4.5$, df = 22, $P = .0002$). The plasma levels of T-treated chicks were well within the range of T levels at this age (see Methods).

Effects of T treatment on immunocompetence.—Detectable antibodies against SRBC were found in only one-third of the gull chicks, which indicated that immunocompetence had not yet developed in most birds of this age. The percentage of birds responding to the immunization did not differ significantly between the groups (C group, 30.4%; T group, 33.3%; χ^2 test, $P = .89$). Therefore, we selected the data from the SRBC competent chicks to test whether the hormonal treatment affects the level of antibody response. It was interesting that the antibody response to SRBC was significantly higher in the T group than in the control group (mean ± SE: C group, 1.6 ± 0.4, $N = 7$; T group, 4.8 ± 0.6, $N = 5$; two-sample *t*-test, $t = 4.5$, df = 10, $P = .001$). This indicates that T treatment enhances immunocompetence.

Experiment B: Hormonal Specificity and Long-Lasting Effects in Chicks

Estrogen and T levels.—Seven days after the start of the hormonal treatment, estrogen levels in blood plasma were elevated only in birds treated with estrogen implants (mean ± SE: C group, 18 ± 4 pg/mL, $N = 10$; E group, 269 ± 46 pg/mL, $N = 5$; DHT group, 47 ± 16 pg/mL, $N = 5$; T group, 55 ± 21 pg/mL, $N = 5$; ANOVA, $F = 20.57$, df = 3, 21, $P < .0001$; two-sample *t*-tests, corrected Ntest = 6, E vs. C, T, or D, $P < .01$, T or DHT vs. C, T vs. DHT, $P > .10$). Levels of T were increased in the T-treated birds and in the DHT-treated birds (mean ± SE, C group, 0.06 ± 0.03 ng/mL, $N = 5$; E group, 0.18 ± 0.12 ng/mL, $N = 5$; DHT group, 0.92 ± 0.29 ng/mL, $N = 4$; T group, 1.58 ± 0.43 ng/mL, $N = 5$; ANOVA, $F = 10.07$, df = 3, 15, $P = .0007$; two-sample *t*-tests,

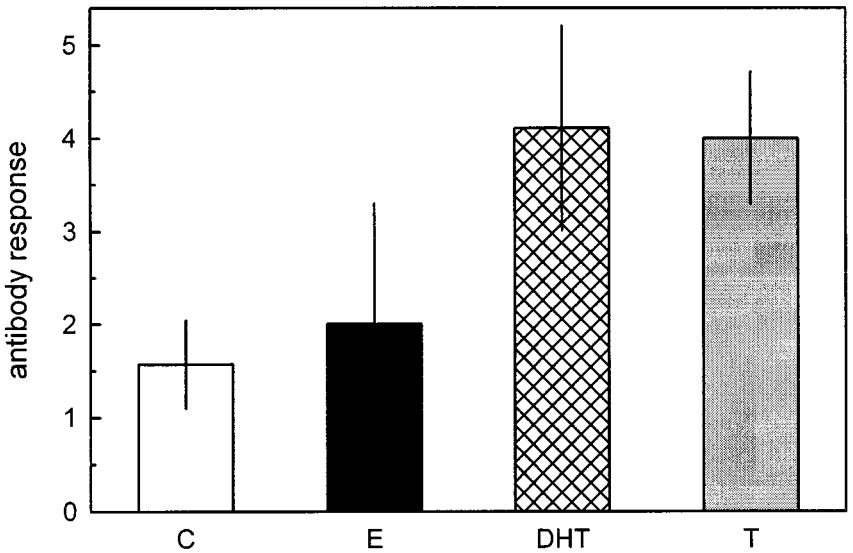

FIG. 2.—Long-term effects of 17β-estradiol (*E; N* = 5), 5α-dihydrotestosterone (*DHT; N* = 9), testosterone (*T; N* = 10), or sham (control) treatment (*C; N* = 14) on the antibody response to SRBC at 40 d of age. Steroid was administered by Silastic tube implants inserted subcutaneously at day 6 of age until day 16 of age, approximately 1 mo before SRBC immunization.

corrected Ntest = 6, T or DHT vs. C, $P < .01$, T or DHT vs. E, $P < .06$, C vs. E, T vs. DHT, $P > .10$). As expected, the T treatment elevated the T level in the blood plasma to the upper part of the physiological range (see Methods). The assay for determining T concentrations has a high cross-reactivity (0.47%) with DHT and was used to determine DHT also. Hence, the increase in T levels observed in this assay in the DHT group almost certainly indicates increased levels of DHT due to DHT treatment.

During the antibody response measurements in 50-d-old fledglings more than a month after removal of the hormonal implants, T levels in blood plasma were very low and in most cases below the detection level of the assay. At this age, no differences existed in T levels between the experimental groups (ANOVA, $F = 0.21$, df = 3, 34, $P = .89$).

Long-term effects of early hormonal treatment on immunocompetence.—Sheep red blood cell immunization caused an increase in antibody titers in 67% of the fledglings. A significant effect of hormonal treatment was found (ANOVA, $F = 2.97$, df = 3, 34, $P = .046$; two-sample *t*-tests, corrected Ntest = 6, T vs. C, $P = .042$; other comparisons, $P > .10$). The birds treated with DHT showed a similar increase in antibody titers as the T-treated birds did, whereas the E-treated birds did not show such an increase (see fig. 2). This indicates that the effect of T on antibody responsiveness is androgen dependent.

No significant effects of sex were found (ANOVA of antibody responses with

treatment and sex as factors, sex, sex × treatment interaction, $P > .10$; mean ± SE antibody responses for males and females, respectively, were the following: T and DHT group, 3.38 ± 1.02 ng/mL, $N = 8$, and 4.55 ± 0.79 ng/mL, $N = 11$; C and E group, 2.4 ± 0.72 ng/mL, $N = 10$, and 0.89 ± 0.48 ng/mL, $N = 9$).

Conclusion

Treatment of black-headed gull chicks with T during the period when the bursa still affects the maturation of lymphocytes mediates an increase in antibody responsiveness (experiments A and B). This effect persists for at least 40 d after the termination of T treatment and is androgen specific (experiment B). Even with the increased levels of T in experiment B relative to experiment A, antibody response was still enhanced.

EFFECTS OF TESTOSTERONE IN 9-MO-OLD JUVENILES

Design

To test the effect of T on immunocompetence at an age when the lymphocytes are likely to be independent of the bursa, we carried out an experiment on juvenile black-headed gulls just entering their first spring (see fig. 1*C*). At this age, juveniles start to perform all adult courtship displays (Groothuis 1989). This therefore provided an excellent basis for studying immunocompetence in relation to the performance of sexual display. Birds were either treated with T or sham operated 1 mo before they would become active in display and while T titers were still low. Experimental groups were kept in separate cages to prevent T-treated birds from becoming dominant over control birds. Antibody responses to a single immunization with SRBC were measured during the experimental treatment. In addition, the energetic consequences of performing display behavior and of producing the antibody response were investigated by measuring changes in body mass during the experiment.

Methods

Rearing conditions.—Birds were collected just before fledging in the field, at the beginning of July. These birds were housed in a large outdoor aviary (4.5 × 4.3 × 2.0 m). Dry food pellets and water were available ad lib. Once every 2 or 3 wk, this was supplemented with mashed hard-boiled eggs. One month before the experiment, the birds were rehoused in groups of five birds in outdoor aviaries of 3.0 × 4.3 × 2.0 m.

Treatment.—A total of 10 birds, housed in two groups of five birds each, were given silicon implants filled with T (length = 12 mm; for details, see Testosterone Treatment of Chicks) and 11 birds, housed in two groups of five and six birds, were sham operated. For details about analyzing T levels by means of radioimmunoassay, see Testosterone Treatment of Chicks. At 10 d after the start of the T treatment, all birds were immunized with SRBC. Because of incomplete data collection, preimmunization antibody titers for 10 birds were assigned as

the mean preimmunization titer for all birds. For further details about methods and assays, see Testosterone Treatment of Chicks.

Sex determination.—To check for sex-specific effects of treatment on humoral immunity, sex was determined by measuring the length of the head plus bill. In fledged birds, this length is greater for males than for females (discriminant value = 8.1 cm) and has a reliability of 95% (Coulson et al. 1983; Koopman 1990). The control group included four males and seven females, and the T group contained four males and six females.

Behavioral observations and body mass measurements.—Behavioral data were collected before immunization. Previous research has shown that the effect of T treatment on agonistic behavior reaches a maximum 5 d after the start of treatment and stays at this level for at least 1 wk (Groothuis and Meeuwissen 1992). Therefore, we made behavioral observations at days 5–7 of T treatment. During these days, the behavior of the gulls was observed for at least 1 h/d/bird during normal within-group interactions (3.5 h/bird in total). The most frequently performed agonistic and sexual displays in black-headed gulls are the oblique and forward (for a definition of these displays, see Groothuis 1989). The oblique is an erect posture accompanied with a loud call (the long call). This display is often followed by the forward, a posture in which the head and neck are held in front of the body and often unaccompanied by a call. Pairs of black-headed gulls can be easily recognized because courtship involves synchronized performance of a ritualized sequence of oblique to forward (see van Rhijn and Groothuis 1987). Total display was defined as the sum of oblique and forward. Frequency of aggression and withdrawal were written down regularly, which gave an indication of dominant relationships.

To measure costs associated with immunocompetence and display performance, we measured the change in body mass by subtracting the body mass at the start of T treatment from the body mass after 2 wk of this treatment. Thus, a negative value refers to a reduction in body mass.

Statistical treatment.—The same statistical procedure was used as described in Testosterone Treatment of Chicks. Measurements of behavior and body mass showed skewed distributions. To obtain normal distributions, body mass was log transformed, and frequency of behavior Poisson ($[X] + ([X + 1])$ transformed, needed for discrete variables sampled per unit of time (see Zar 1984).

Results

Testosterone levels.—The average titer of T after treatment at the start of the immunization with SRBC was 2.42 ng/mL (SE = 0.40 ng/mL, $N = 10$, range = 0.56–5.16 ng/mL). This value is low in comparison with the T level of 5 ng/mL of adult males at the start of the breeding season in May (Groothuis and Meeuwissen 1992). Testosterone titer in the T group was significantly higher than that of control birds, which had T titers near the detection level of the radioimmunoassay (mean ± SE, 0.09 ± 0.03 ng/mL, $N = 11$, range = <0.05–0.36 ng/mL; two-sample *t*-test, $t = 9.98$, df = 19, $P < .0001$). No sex difference in T levels was found within the T group (two-sample *t*-test: $t = 1.64$, df = 8, $P = .14$).

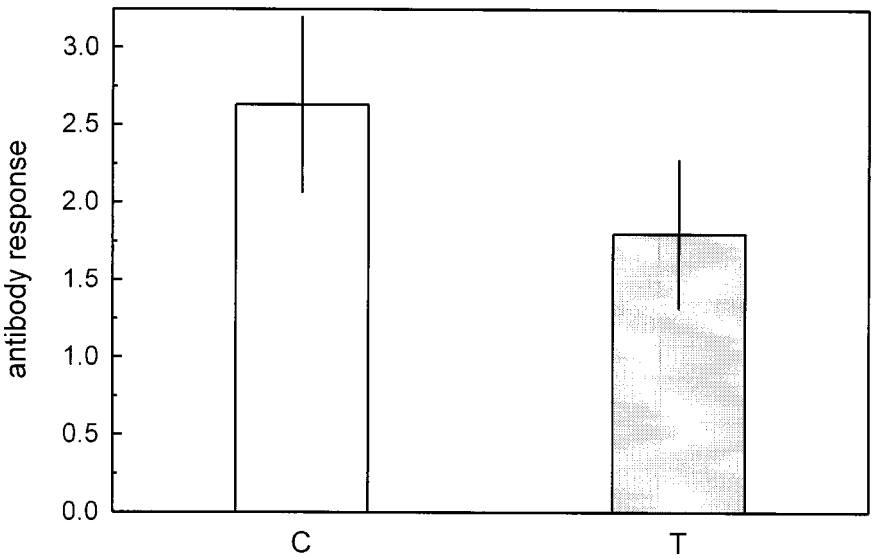

FIG. 3.—Effect of a testosterone treatment (*T; N* = 10) or sham (control) treatment (*C; N* = 11) on the antibody response to SRBC at 9 mo of age. Testosterone was administered by Silastic tube implants 10 d prior to immunization.

Effects of T treatment on immunocompetence.—Ninety-five percent of the gulls responded to SRBC immunization by elevating antibody titers. To test the effect of T treatment on antibody titers, an ANOVA was carried out with hormonal treatment and sex as factors. Females tended to have higher antibody titers than males, but this was not significant (sex, $F = 3.59$, df = 1, 14, $P = .079$; sex × treatment, $F = 0.00$, df = 1, 14, $P = .97$; mean ± SE antibody responses for males and females, respectively, were the following: T group, 1.03 ± 0.66 ng/mL, $N = 4$, and 2.32 ± 0.61 ng/mL, $N = 6$; C group, 1.65 ± 0.48 ng/mL, $N = 4$, and 3.19 ± 0.79 ng/mL, $N = 7$). In contrast to the results of the experiments with chicks, T did not enhance antibody titers. Instead, T-treated birds showed 31% lower antibody titers than did C birds (fig. 3). An ANOVA with treatment and sex as factors showed that this difference between the experimental groups was not significant (treatment, $F = 1.13$, df = 1, 14, $P = .31$). However, because of low sample size and high variation, the power of the test was very low. To have a power of 80% for detecting a 31% suppression of antibody titers, the *N* value in both experimental groups would need to be 70 birds.

We also tested whether a correlation existed between the individual scores of T levels and antibody titers within the T group. This correlation was far from significant ($R^2 = 0.075$, $N = 10$, NS; corrected Ntest = 3).

Effects of T treatment on display behavior and body mass.—Testosterone-treated birds showed a strong increase in the frequency of display behavior compared with control birds (two-sample *t*-test, $t = 6.18$, df = 18, $P < .0001$). At

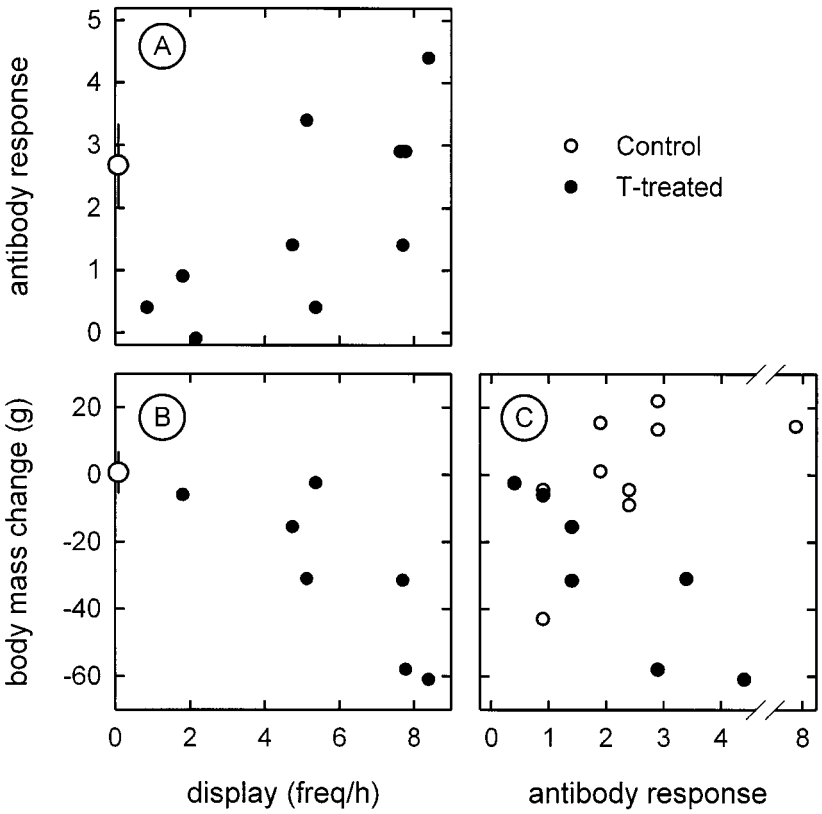

FIG. 4.—Correlations between individual scores of the antibody response to SRBC immunization, frequency of display, and change in body mass during testosterone treatment; means ± SE of control birds are given in *A* and *B*. In this experiment, testosterone-treated birds (*T; N* = 10) were housed separately from control birds (*C; N* = 11). Some data points are missing because they were not measured.

the same time, body mass was significantly decreased in T birds compared with that of C birds (two-sample *t*-test, $t = 2.90$, df = 14, $P = .012$). Variation in both measurements was largest within the T group. The variation in the T group could not be explained with individual T levels, perhaps because of the limited sample size (display, $R^2 = 0.19$, $N = 10$, NS; body mass change, $R^2 = 0.49$, $N = 7$, NS; corrected Ntest = 3).

The relation among antibody titers, display behavior, and body mass.— Within the T group, the individual scores of antibody titers showed a positive correlation with the frequency of display (fig. 4*A*) ($R^2 = 0.51$, $N = 10$, $P = .040$; corrected Ntest = 3). Both antibody titers and display behavior were negatively correlated with changes in body mass (fig. 4*B, C)* (display, $R^2 = 0.59$, $N = 7$, $P = .044$; antibody titers, $R^2 = 0.80$, $N = 7$, $P = .020$; corrected Ntest = 3).

The large individual differences in antibody titers between birds might be related to the social rank of the birds. Testosterone treatment increased the level of competition between birds, and T birds that performed the fewest display were clearly the subdominant birds in the cage. In contrast to those in the T group, antibody titers in the C group did not correlate significantly with body mass changes (fig. 4*C*) ($R^2 = 0.23$, $N = 9$, NS).

Conclusion

Testosterone treatment did not affect antibody titers in juvenile birds. The data suggest that in the groups in which the birds were treated with T, the level of competition increased, leading to clear individual differences in dominance, frequency of display, and reduced body mass. It is interesting these differences were correlated with differences in antibody titers. In comparison with subdominant birds, the dominant birds had higher frequencies of display and higher antibody titers but also lost more body mass. The data suggest that the high frequency of display behavior, possibly in combination with a high level of antibody production, is energetically costly.

DISCUSSION

This study shows that T levels, which were experimentally increased in 2-wk-old black-headed gull chicks to the maximum of the physiological range, enhanced antibody responses to SRBC immunization. This effect persisted for at least 40 d after the end of hormonal treatment and was androgen specific given that both T and DHT treatment increased immune responsiveness while estrogen did not. This study also shows that the effect of T on immune responsiveness is age specific because experimentally increased T levels in juvenile black-headed gulls did not enhance antibody responsiveness. In agreement with mammalian studies and the supposedly immunosuppressive effect of T (Grossman 1985), there was a tendency, but not a significant one, for females to show a somewhat higher antibody response than males. Overall no statistical significant differences in antibody titers were found between the sexes at any age, which might be due to the low sample sizes. A lack of sexual differences is in accordance with the general scarcity of sexual dimorphism in this species (van Rhijn 1985).

From this study, we deduce that in chicks and juveniles of the black-headed gull, testosterone, increased to the level at which agonistic and courtship display performance is facilitated, does not have negative fitness costs in terms of decreased immune function, at least in ad lib. food conditions. In the only two studies we are aware of in which T levels were manipulated to test the immunocompetence handicap hypothesis, an increase in the number of ectoparasites was found (Saino et al. 1995; Salvador et al. 1996). These results seem to indicate an immunosuppressive effect of T, in contradiction to our results. However, this discrepancy might be explained in several ways. First, we may have tested another part of the immune system, namely, the humoral response to internal infection, while the other studies found an effect on ectoparasites. Second, the increase in ectoparasite load might be caused not by an immunosuppressive effect

of T but by other mechanisms such as a higher exposure to parasites by a T-induced change in behavior or T-induced favorable conditions for the parasites such as an increase in peripheral blood flow.

The result that physiological levels of T enhance antibody production in chicks (also found for domestic fowl; Deyhim et al. 1992) but not in birds that are almost sexually mature may be explained from life-history theory. In both young and adult birds, T facilitates visual and postural displays that play a role in competitive interactions (Balthazart 1983; Schwabl 1993; Wingfield et al. 1990). When competition increases, the need for immunocompetence also increases because of the higher likelihood of injuries and contact with other birds that might carry infections. A positive correlation between T (-mediated display) and immunocompetence, as we found for black-headed gull chicks, would thus be adaptive. However, an important difference between adults and young birds is that competition in young birds serves a direct survival function, whereas in adults it has an additional function in courtship. Despite negative effects on survival, adults but not young birds may benefit from reallocating resources from the immune system to the reproductive system because this increases their reproductive output. Testosterone may play an important role in regulating this process (Folstad and Karter 1992).

The result that treatment of black-headed gulls did not suppress antibody responsiveness at any age was somewhat surprising because androgens are generally regarded as immunosuppressive (Grossman 1984, 1985). Experiments with chicks of the domestic fowl showed that, especially early in ontogeny, T can affect the immune system via the bursa of Fabricius. This organ plays an important role in affinity maturation: the switch from immunoglobulin M (IgM) production by lymphocytes to production of immunoglobulin G (IgG) (Lerner et al. 1971; Glick 1983). When challenged with antigens, cells that have undergone affinity maturation will be stimulated by antigens over a longer period and have more chance of developing into memory cells. Testosterone treatment of both embryos and chicks of the domestic fowl causes a strong regression of the bursa coinciding with migration of lymphocytes out of the bursa (Lerner et al. 1971; Glick 1983; Ratcliffe 1989; Deyhim et al. 1992; Fennell and Scanes 1992). In agreement with the role of the bursa in affinity maturation, T treatment in young birds has a strong negative effect on IgG production (Lerner et al. 1971; Deyhim et al. 1992). Testosterone-treated chicks have higher IgM levels than do control chicks. These higher IgM levels in T-treated chicks may be a consequence of a higher proportion of IgM- versus IgG-producing lymphocytes due to the inhibition of affinity maturation (Deyhim et al. 1992). Early exposure to T may thus affect the quality of the immune system.

Our study shows that during postnatal growth, high levels of T, which are still within the physiological range, lead to long-term enhancement of antibody responsiveness. This effect of T is age specific since T treatment of juveniles did not affect antibody responsiveness. We only measured primary responses to SRBC immunization. The antibody levels are thus mainly IgM production. The enhancement found for T treatment early in ontogeny may thus be due to the inhibition of affinity maturation. Persistence of the effects of an early treatment

with androgens, especially long after hormone levels have returned to the baseline, may be related to the cellular life span of the lymphocytes that had developed in the T-treated birds. This suggests that the endogenous increased levels of T of territorial black-headed gull chicks may have important functional consequences for the immunocompetence of adult birds. In juveniles, T can no longer affect lymphocyte populations via its effect on the bursa, because at this stage most lymphocytes have already migrated from this gland. This might explain the lack of effect we found of T on the antibody production in 9-mo-old juvenile gulls.

Although T treatment of juvenile gulls did not significantly affect antibody responsiveness, T-treated juveniles that showed relatively low frequencies of display behavior had suppressed antibody responses in comparison with actively displaying T-treated juveniles. Testosterone-treated juveniles showing low frequencies of display seemed to be subdominant. Moreover, since during agonistic interactions display is often followed by aggression by the displaying bird and withdrawal of the opponent, even in cases in which no aggression takes place (T. G. G. Groothuis and A. F. H. Ros, unpublished manuscript), the frequency of display might be used as an indication of dominance. This was confirmed by anecdotal records. From this it follows that social rank was positively related to antibody responsiveness. Social stress resulting from low social status may have increased the concentration of free corticosterone in the blood, which is a potent suppressor of the immune system (see the review in Bohus and Koolhaas 1993). In the control groups, antibody responsiveness was not suppressed because these birds did not perform any display and hence had no clear social rank order. Thus, low social status may be a causal factor in reducing antibody responsiveness. This is currently under investigation.

In the T-treated juvenile gulls, antibody titers were negatively correlated with changes in body mass indicating energetic consequences of high antibody responsiveness. However, no such correlation was found in the behaviorally inactive control group, which suggests that the energetic demands of high immunocompetence can be compensated for. Testosterone treatment strongly increased the frequency of display behavior. Gulls expressing these postural displays more frequently showed a considerable decrease in body mass, which indicates that displaying is costly (see also van Rhijn 1980). Despite this decrease in body mass, birds that displayed frequently also had higher antibody titers. Display behavior may thus signal the quality of the bird in terms of immunocompetence. This correlation is consistent with the Hamilton and Zuk (1982) hypothesis that displays are a handicap revealing parasite resistance.

We tested the primary antibody response to a challenge with SRBC, which is only one measure of many aspects of immunocompetence, and have no data on how T affects other aspects of immunocompetence. Nevertheless, this study demonstrates that in the black-headed gull, high levels of T, still within the physiological range, can facilitate display behavior without suppressing the immune system. There is, of course, the possibility that higher doses of T would have resulted in immunosuppression. However, a 1.5 higher dose did not have such a result in juvenile gulls (T. G. G. Groothuis, P. Korsten, and L. Zwigge-

laar, unpublished data). Therefore, our results contradict one of the major premises of the immunocompetence handicap hypothesis (Folstad and Karter 1992; but see modifications in Wedekind and Folstad 1994).

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